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Dear Editor,

I send you a copy of revised manuscript entitled “BlaB-15, a new BlaB metallo- β -lactamase variant found in an *Elizabethkingia miricola* clinical isolate” by Colapietro M et al. DMID-15-596-R1 that I would like to submit on Diagnostic Microbiology and Infectious Disease.

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Thank you in advance for your cooperation.

Sincerely yours,

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Response to reviewer comments:

Page 3, line 17. Has been corrected

Page 3, line 35. Has been corrected

Page 3, Line 45. Has been corrected

Page 4, Line 27. Has been corrected

Page 4, Lines 42-45. The sentence has been deleted

Page 5, Line 14. Has been deleted

Page 5, Line 19. The GOB-7 enzyme reported in GenBank lacks in C-terminal portion.

Page 5, Line 29. A sentence explaining why the blaGOB-like gene was not sequenced has been inserted.

Page 5, Line 54. Has been corrected

Page 6, Line 1. Has been corrected

Page 6, Line 5. Has been corrected

Page 6, Lines 16-18. The sentence has been deleted.

Page 6, Lines 20-22. The sentence has been corrected.

Table 1. Has been corrected as suggested.

BlaB-15, a new BlaB metallo-β-lactamase variant found in an *Elizabethkingia miricola* clinical isolate

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Keywords: *Elizabethkingia miricola*, BlaB, Metallo-β-lactamase, MBL, GOB

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Abstract

A multi-drug resistant strain of *E. miricola* was isolated from the urine of a 2-year old boy hospitalized for severe clinical conditions. The strain produces two metallo- β -lactamases belonging to subclasses B1 and B3: a new BlaB variant (BlaB-15) and a GOB-7-like enzyme.

Genus *Elizabethkingia* (formerly *Chryseobacterium*) belongs to *Flavobacteriaceae* family and includes two main species, *Elizabethkingia meningoseptica* and *Elizabethkingia miricola*, which are genetically different from other *Elizabethkingia* species, as revealed by 16S rRNA gene sequence studies (Kim et al., 2005). These bacteria are ubiquitous in natural (freshwater, saltwater and soil) and hospital environments. *E. meningoseptica* is an important emerging pathogen responsible for severe healthcare infections (Jean et al., 2014). In clinical settings, *Elizabethkingia* species have been described as etiological agents of meningitis, especially in premature infants, sepsis, bacteremia, pneumonia and endocarditis (Jean et al., 2014; de Silva et al., 2013). Carbapenem resistance in *E. meningoseptica* is mainly due to the production of metallo- β -lactamases (MBLs) as GOB and BlaB variants (Bellais et al., 2000; Gonzales and Vila, 2012).

E. miricola was isolated for the first time in 2003 from the condensation water on the space station *Mir* (Li et al., 2003). The first case of infection due to *E. miricola* (pneumonia and sepsis) was described in 2008 in a man with lymphoma and under mechanical ventilator support (Green et al., 2008). In the present study we identified a new BlaB variant (BlaB-15) from *E. miricola* isolated from a children with a complicated clinical condition. In September 2012, a 2-year old boy was admitted to the pediatric Emergency Department of the Children Bern University Hospital with fever and severe clinical conditions due to his underlying diseases (i.e., spina bifida, bladder exstrophy, atelectasis, tracheostomy, chronic kidney insufficiency, and Mitrofanoff stoma fistula). Urine was collected from an intermittent urinary catheter and showed leucocyte count of 500 cells/ μ l and presence of nitrites. In addition, the urine sample gave the following results: 10.000 CFU/ml of *Klebsiella pneumoniae* (ESBL-positive), 10.000 CFU/ml of *E. miricola*, 100.000 CFU/ml of *Pseudomonas aeruginosa*, 100 CFU/ml of *Candida* and 100 CFU/ml of viridans streptococci. The young patient was under long-term treatment with nitrofurantoin.

The aim of this study was to characterize the BlaB-15 enzyme and to evaluate its contribution to resistance phenotype. *E. miricola* strain SW-2 was identified by using MALDI TOF MS system (Bruker Daltonik, Germany). Antimicrobial susceptibility test was performed by microdilution in cation-adjusted Mueller–Hinton broth (BBL, Becton Dickinson) using Sensititre ESB1F plates and GNX2F (TREK Diagnostic Systems, West Sussex, UK). Susceptibility results were interpreted according to current European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria set for *Pseudomonas* spp. (Eucast, 2015). As shown in Table 1, *E. miricola* SW-2 exhibits high MIC values for a large panel of antimicrobials including all cephalosporins, aztreonam, carbapenems, aminoglycosides, fluoroquinolones and polymyxins.

Genomic DNA of *E. miricola* SW-2 was extracted according to standard procedure (Sambrook et al., 1989). The presence of large plasmid was also ascertained using Kado method (Kado and Liu, 1981), but no plasmid bands were observed in *E. miricola* SW2. PCR screening was performed using specific primers to identify *bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM-1}, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} determinants as previously reported (Perilli et al., 2013); the presence of *blaB* and *bla*_{GOB} genes was explored by using the following primers: BlaB-for 5'GGGGCATATGTTGAAAAGATTAAAAGGATTATT, BlaB-rev 5'GGGGGATCCTTAATTTGAAGCCTTTTGTGTTTGTG, BlaGOB-for 5'ATGAGAAATTTTGCTACACTG and BlaGOB-rev 5'TCGAACTGACTTGTCATG. Two amplicons of 747 bp and 825 bp were amplified by BlaB-for/BlaB-rev and BlaGOB-for/BlaGOB-rev primers, respectively. The purified fragments were sequenced on both strands using a BigDye Sequencing Reaction Kit and an ABI PRISM1 310 Capillary Automated Sequencer (Life Technologies, Monza, Italy).

Nucleotide sequences revealed that amplicons of 747 bp and 825 bp encoded for *blaB*-like and *blaGOB*-like metallo- β -lactamases (MBLs), respectively. The amplicon of 747 bp, encoding for an

open reading frame of 249 amino acids, had more than 90% amino acid identity with BlaB variant. However, some changes in the amino acid sequence make the present BlaB enzyme as new variant, named BlaB-15 (GenBank accession number KR054962). The figure 1 illustrates the comparison of BlaB-15 with other BlaB-variants with which our enzyme shows an higher similarity score (BlaB, BlaB-5, BlaB-7 and BlaB-8). BlaB-15 shows 18 different amino acids in the mature enzyme and 8 different residues in signal peptide. The gene *bla*GOB-like of 825 bp encodes for a protein of 280 amino acids that lacks the C-terminal portion. In figure 2 our GOB enzyme was compared with GOB-1 and GOB-7 variants. Compared to GOB-1, GOB-like shows 19 different residues in the mature enzyme. Despite of GOB-7, the enzyme produced by *E. miricola* SW2 showed an unusual residue of valine at position 258 (BBL B3 classification). Among GOB MBLs (<http://www.ncbi.nlm.nih.gov>), the position 258 is usually occupied by a leucine (GOB-1, -3, -4, -5, -6, -12, -13) or isoleucine (GOB-8, -9, -10, -18) residues (Bellais et al., 2000). However, the *bla*GOB-like gene found in *E. miricola* was not cloned and GOB-like enzyme was not characterized because it lacks in C-terminal region.

The *bla*B-15 gene was cloned into pET-24(a) vector using *Nde*I and *Bam*HI restriction sites to obtain the recombinant plasmid pET-BlaB-15 that was inserted by transformation in *E. coli* BL21(DE3) competent cells. The authenticity of recombinant plasmid was verified by DNA sequencing. The contribution of BlaB-15 to resistance phenotype was investigated versus a large panel of β -lactams by Minimal Inhibitory Concentration (MIC) experiments using *E. coli* BL21(DE3)/pET-BlaB-15. The antimicrobial susceptibility test was performed by conventional microdilution broth procedure, using a bacterial inoculum of 5×10^5 CFU/ml as recommended by CLSI (CLSI, 2006). As shown in table 1, BlaB-15 confers resistance to penicillins- β -lactamase inhibitors combinations, cefotaxime, cefoxitin and carbapenems. Comparing MIC values with that

1 reported by BlaB prototype (Bellais et al., 2000), BlaB-15 seems to confer a broader resistance
2 phenotype against cephalosporins.
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5 In this study, we described the simultaneous presence in *E. miricola* of BlaB-15, a new BlaB variant
6 belonging to subclass B1 MBL, and GOB-7-like enzyme belonging to subclass B3 MBL. To date,
7
8 BlaB and GOB MBLs were mainly found in *E. meningosepticum* (Bellais et al., 2000). To our
9
10 knowledge, this is the first finding of metallo- β -lactamases in *E. miricola* clinical strains.
11
12 Additionally, the 2-year old baby was also colonized by an ESBL-producing *K. pneumoniae*. The
13
14 co-colonization/infection of a patient with different strains producing several β -lactamases
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16 complicates treatment.
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Legend to the figures

Figure 1

The MBL BlaB-15 amino acid sequence (GenBank accession number KR054962) was compared with BlaB prototype (GenBank accession number O08498), BlaB-5 (GenBank accession number Q9KJA9), BlaB-7 (GenBank accession number Q9KJA8) and BlaB-8 (GenBank accession number Q9KJA7). Dashed lines indicate identical amino acid. The residues of BlaB-5, BlaB-7, BlaB-8 and BlaB-14 that differs from BlaB are also indicated. Arrow indicate the first residue of N-terminal region. In bold is indicated the sequence of BlaB-15 enzyme found in *E. miricola* (this study). The catalytic residues including Zn1 ligand (H116, H118, H196) and Zn2 ligand (D120, C221, H263) are in bold underlined.

Figure 2

The GOB-like was compared with GOB-1 (AF090141) and GOB-7 (AF189297). Dashed lines indicate identical amino acids. In bold is indicated the sequence of GOB-like enzyme found in *E. miricola* (this study). The residues of GOB-7 and GOB-like that differs from GOB-1 are also indicated. The box shows the unique different residue between GOB-7 and GOB-like.

Figure 1

Figure 1: Amino acid comparison of the BlaB-15 MBL with other BlaB, BlaB-5, BlaB-7 and BlaB-8 variants

		↓
BlaB	MLKKIKISLILALGLTSLQAFGQENPDVKIEKLDNLYVYTTYNTFNGTKYAANAVYLV	
blaB-5	---RL-GL-V----F-G-Q---Q---I-----K-----M--	
blaB-7	-M-GL-GL-V----F-G-Q---Q---I-----K-----M--	
blaB-8	-M-GL-GL-V----F-G-QV---Q---I-----K-----M--	
blaB-15	---RL-GL-V----F-G-QV---Q---I-----K-----M--	
	:* :* *:****:*.**.**:***:*****:*****:*	
BlaB	DKGVVIDCPWGEDKFKSFTDEIYKKHGKKVIMNIATHSHHDDRAGGLEIFYGKIGAKTYST	
blaB-5	-----S-----L-----	
blaB-7	---I---S-----L-----	
blaB-8	-----S-----L-----	
blaB-15	-----S-----L-----	
	:.*****:*****:*****:*****	
BlaB	KMTDSILAKENKPRAQYTFDNNKSFKVGKSEFQVYYPGKGHTADNVVVWFPEKVLVGGC	
blaB-5	-----K-----NT-----D-----	
blaB-7	-----K-----T-----D-----	
blaB-8	-----K-----T-----D-----	
blaB-15	-----K-----T-----D-----	
	*****:*****:*****:*****:*****	
BlaB	IIKSADSKDLGYIGEAYVNDWTQSVHNIQQKFSGAQYVVAGHDDWKDQRSIQHTLDLINE	
blaB-5	-V--G-----I-----PDV-----T-----SD	
blaB-7	-V--G-----F-----I-----PDV-----T-----S-	
blaB-8	-V--G-----F-----I-----PDV-----T-----S-	
blaB-15	-V--G-----I-----PDV-----T-----SD	
	*:**.*****:*****:*****...*****:*****:	
BlaB	YQQKQKASN	
blaB-5	-----	
blaB-7	-----	
blaB-8	-----	
blaB-15	-----	

Figure 2

Figure 2: Amino acid comparison of the GOB-like with GOB-1 and GOB-7 variants

GOB-1	MRNFATLFFMFICLGLNAQVVKEPENMPKEWNQTYEPFRIAGNLYYVGTYDLASYLIVTD
GOB-7	-----
GOB-like	-----LLV--S-----
GOB-1	KGNILINTGTAESLPIIKANIQKLGFNKYKDIKILLTQAHYDHTGALQDLKTETGAKFYA
GOB-7	-----F---G-----F-----
GOB-like	-----F---G-----F-----
GOB-1	DKADADVLRRTGGKSDYEMGKYGVTFKPVTPDKTLKDQDKITLGNTILTLLHHPGHTKGSC
GOB-7	-----L-----T-----
GOB-like	-----L-----T-----
GOB-1	SFIFETKDEKRKYRVLIANMPSVIVDKKFSEVTAYPNIQSDYAYTFKAMKNLDFDLWVAS
GOB-7	-----N---KI-----I-----G-----I-----
GOB-like	-----N---KI-----I-----G-----V-----
GOB-1	HASQFDLHEKRKEGDPYNPQLFMDKQSYFQNLNDLEKSYLDKIKKDSQDK
GOB-7	-----T-----S-----EN--KRLE
GOB-like	-----T-----S-----EN--KRLE

Table 1: Antimicrobial susceptibility pattern of *E. miricola* SW2 and *E. coli* BL21(DE3)/pET-BLAB-15

Antibiotic	<i>E. miricola</i> SW2 [§]	<i>E. coli</i> BL21(DE3) pET-BLAB-15 [*]	<i>E. coli</i> BL21(DE3) [*]
Piperacillin-tazobactam	8	>64	0.25
Ticarcillin-clavulanate	≥256	>64	0.25
Cefoxitin	64	16	0.12
Ceftriaxone	64	4	0.12
Cefotaxime	32	16	0.50
Ceftazidime	128	2	0.12
Cefepime	16	0.50	0.06
Aztreonam	≥32	0.12	0.06
Imipenem	≥32	16	0.12
Meropenem	≥16	16	0.12
Ertapenem	≥8	8	0.12
Gentamicin	<0.5	-	-
Tobramycin	≥16	-	-
Amikacin	≤2	-	-
Ciprofloxacin	0.50	-	-
Levofloxacin	<0.50	-	-
Trimethoprim/sulfamethoxazole	4	-	-
Colistin	≥8	-	-
Polymixin B	≥8	-	-
Doxycycline	≤1	-	-
Minocycline	≤1	-	-
Tigecycline	0.50	-	-

[§]Antimicrobial susceptibility tests were performed by microdilution in cation-adjusted Mueller–Hinton broth (BBL, Becton Dickinson) using Sensititre ESB1F plates and GNX2F

^{*}The antimicrobial susceptibility test was performed by the conventional microdilution broth procedure, using a bacterial inoculum of 5×10^5 CFU/ml as recommended by CLSI (CLSI, 2006). MIC values are expressed in µg/ml.

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Abstract

A multi-drug resistant strain of *E. miricola* was isolated from the urine of a 2-year old boy hospitalized for severe clinical conditions. The strain produces two metallo- β -lactamases belonging to subclasses B1 and B3: a new BlaB variant (BlaB-15) and a GOB-7-like enzyme.

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Reaction Kit and an ABI PRISM1 310 Capillary Automated Sequencer (Life Technologies, Monza, Italy).

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microdilution broth procedure, using a bacterial inoculum of 5×10^5 CFU/ml as recommended by CLSI (CLSI, 2006). As shown in table 1, BlaB-15 confers resistance to penicillins- β -lactamase /inhibitors combinations, cefotaxime, ceftazidime and carbapenems. Comparing MIC values with that reported by BlaB prototype (Bellais et al., 2000), BlaB-15 seems to confer a broader resistance phenotype against~~have a more large phenotype spectrum versus~~ cephalosporins.

In this study, we described the simultaneous~~ly~~ presence in *E. miricola* of BlaB-15, a new BlaB variant belonging to subclass B1 MBL, and GOB-7-like enzyme belonging to subclass B3 MBL. To date, BlaB and GOB MBLs were mainly found in *E. meningosepticum* (Bellais et al., 2000). To our knowledge, this is the first finding of metallo- β -lactamases in *E. miricola* clinical strains. Additionally, the 2-year old baby was also colonized by an ESBL-producing *K. pneumoniae*. ~~In fact, this strain was also investigated (data not shown) and a CTX-M-15 enzyme was found in the chromosomal DNA of *K. pneumoniae*.~~ The co-colonization/infection of a patient with different strains producing several β -lactamases ~~makes difficult the infection~~complicates treatment.

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Legend to the figures

Figure 1

The MBL BlaB-15 amino acid sequence (GenBank accession number KR054962) was compared with BlaB prototype (GenBank accession number O08498), BlaB-5 (GenBank accession number Q9KJA9), BlaB-7 (GenBank accession number Q9KJA8) and BlaB-8 (GenBank accession number Q9KJA7). Dashed lines indicate identical amino acid. The residues of BlaB-5, BlaB-7, BlaB-8 and BlaB-14 that differs from BlaB are also indicated. Arrow indicate the first residue of N-terminal region. In bold is indicated the sequence of BlaB-15 enzyme found in *E. miricola* (this study). The catalytic residues including Zn1 ligand (H116, H118, H196) and Zn2 ligand (D120, C221, H263) are in bold underlined.

Figure 2

The GOB-like was compared with GOB-1 (AF090141) and GOB-7 (AF189297). Dashed lines indicate identical amino acids. In bold is indicated the sequence of GOB-like enzyme found in *E. miricola* (this study). The residues of GOB-7 and GOB-like that differs from GOB-1 are also indicated. The box shows the unique different residue between GOB-7 and GOB-like.